lipid. Basis for this clarification can be found on page 21, lines 24-29.

II. Rejection Under 35 U.S.C. §112, second paragraph

Claims 8, 9, and 11-19 were rejected under 35 U.S.C. §112, second paragraph as allegedly indefinite. Specifically, the Examiner objected to the word "including."

Claims 8 and 13 having been amended as described above, the current rejection is no longer applicable. Withdrawal of the rejection under 35 U.S.C. §112, second paragraph is respectfully requested.

III. Rejection Under 35 U.S.C. §103

Claims 8-19 were rejected under 35 U.S.C. §103 as allegedly obvious over Janoff *et al.* (U.S. Patent No. 4,897,384) or Popescu *et al.* (U.S. Patent No. 4,981,692) in view of Yoshioka *et al.* (U.S. Patent No. 5,593,622).

This rejection is respectfully traversed.

A. Summary of the Present Invention

The invention is directed to a liposome composition for treating a systemic infection which is localized at a site other than the fixed macrophages residing in the liver or the spleen. The composition comprises liposomes formed of a vesicle forming lipid and of a polymer derivatized vesicle-forming lipid to provide the liposomes with an extended blood circulation lifetime. Entrapped in the liposomes is a therapeutic agent having activity against the pathogen causing the infection. The liposomes are sized of between about 0.07-0.20 μm in diameter and are able to accumulate in the infected tissue following intraveneous administration.

B. Summary of the Cited References

JANOFF *ET AL.* describe a composition for reducing the toxicity of therapeutic drugs. The composition consists of a complex between a drug and a ligand, where the ligand is selected according to its ability to prevent or reduce binding of the drug to the *in vivo* drug toxicity receptor (Col. 7, lines 37-42). Janoff *et al.*

speculate that the ligand, by forming a complex with the drug, prevents binding of the drug to the toxicity receptor *in vivo* (Col. 9, lines 53-55) or that the ligand itself binds to the toxicity receptor thereby preventing drug interaction with the receptor (Col. 10, lines 2-4).

Janoff *et al.* describes four embodiments of drug/lipid-ligand complexes:

(1) aminoglycoside antibiotics (*e.g.*, gentamycin) complexed with phosphatidylinositol bisphosphate;

(2) amphotericin B complexed with a sterol;

(3) adriamycin complexed with cardiolipin; and

(4) cisplatin complexed with phosphatidylinositol bisphosphate.

In each embodiment, the lipid must either (1) form a complex with the drug to prevent binding of the drug to the endogeneous receptor (Col. 9, lines 53-55) or (2) bind to the endogeneous toxicity receptor itself to prevent the drug from binding to the receptor (Col. 10, lines 2-4). Janoff *et al.* note that for cardiolipin, the head group of the lipid interacts with the toxicity receptor and the drug (Col. 12, lines 51-57). Interaction would also be expected to occur at the head group of phosphatidylinositol bisphosphate, since it is in the head group where the polar moieties are located (see structure of phosphatidylinositol bisphosphate in ATTACHMENT 1).

POPESCU ET AL. describe antibiotic-containing liposomes for treatment of infections which reside in the reticuloendothelial system (RES), specifically in macrophages (Col. 4, lines 49-64). As such, the liposome composition described by Popescu is intended to be, after *in vivo* administration, taken up by phagocytic cells of the RES (Col. 5, lines 2-10).

Popsecu *et al.* do not show liposomes which include a vesicle-forming lipid derivatized with a polymer chain, such as polyethylene glycol (PEG). Nor would it make sense to modify Popescu *et al.* to include such derivatized lipids, since it is well know in the liposome art that liposomes having a surface coating of PEG avoid uptake by the RES (see, for example, U.S. Patent No. 5,013,556).

Avoiding uptake by the RES is contrary to the purpose of the liposome composition of Popescu *et al.*

YOSHIOKA *ET AL.* disclose PEG-derivatized phospholipids for inhibiting adsorption of proteins on the surface of a liposome.

C. Analysis: Rejection over Janoff *et al.* in view of Yoshioka *et al.*

In determining obviousness, "it is impermissible within the framework of section 103 to pick and choose from any one reference only so much of it as will support a given position to the exclusion of other parts necessary to the full appreciation of what such reference fairly suggests to one skilled in the art."[1]

Applicants respectfully submit the Examiner has not given fair weight to what Janoff *et al.* teach in its entirety. The Examiner characterizes Janoff *et al.* as teaching "gentamycin-containing liposomes (note the abstract, examples and claims)." The Examiner also asserts that "Janoff on columns 15-17 teaches liposomes containing PEG-cholesterol also and according to Janoff even these preparations have reduced toxicity (noted col. 17)." (Office Action at pages 3-4 dated December 6, 1999).

As described in detail above, Janoff *et al.* is concerned with reducing toxicity of drugs by complexing the drug with a lipid ligand that corresponds to the drugs toxicity receptor *in vivo*. The lipid ligand in the complex must either complex with the drug, rendering the drug unavailable for interaction with the toxicity receptor *in vivo*, or the lipid ligand must itself interact with the toxicity receptor *in vivo* and thereby inhibit interaction between the drug and the toxicity receptor.

In one embodiment of Janoff *et al.*, the lipid ligand phosphatidylinositol bisphosphate and the drug gentamycin are described as forming a complex or a liposome. To fulfill the teaching in accord with Janoff *et al.*, the lipid ligand must be capable of interaction with the drug and/or the *in vivo* toxicity

[1] In *Bausch & Lomb v. Barnes-Hind/Hydrocurve* 796 F.2d 443, 230 USPQ 416, Fed. Cir. (1986).

receptor for the drug.

The Examiner suggests that it is obvious, in light of Yoshioka *et al.*, to derivatize the phosphatidylinositol bisphosphate lipid ligand of Janoff *et al.* with a PEG chain in order to prevent the liposomes from aggregating, as taught by Yoshioka *et al.*

In making this suggested modification, the Examiner ignores the necessary features of Janoff *et al.* Namely, that the phosphatidylinositol bisphosphate lipid must be capable of interaction with the drug and/or the *in vivo* toxicity receptor for the drug. Interaction with the drug and/or the toxicity receptor occurs with the head group of the lipid (Col. 9, line 52-Col. 10, line 2). Modification of the lipid with a PEG chain must also occur at the head group of the lipid since the possible reactive moieties are localized in the head group.

It is well recognized in the art that conjugation of a PEG chain to a lipid head group forms a steric " shell" about the lipid head group. Such a shell is depicted in Figs. 3A-3B of Hristove *et al.*[2] Such a shell is known to hinder binding interaction between the lipid head group and a receptor, as described by Noppl-Simson and Needham.[3]

The Examiner points to the disclosure of Janoff *et al.* at Cols. 15-17 of a PEG-cholesterol derivative. In this embodiment, PEG acts to solubilize the cholesterol to provide a clear solution for injection (Col. 11, lines 57-59). Contrary to the Examiner's assertion that Janoff *et al.* teach PEG-cholesterol liposomes, liposomes are explicitly excluded for use in this embodiment (Col. 11 line 65-Col. 12, line 6). The cholesterol must be available to interact with the drug and/or the toxicity receptor *in vivo*. Cholesterol anchored in a lipid bilayer is not exposed for interaction. Modification of the active hydroxyl moiety on the cholesterol with a PEG chain sterically hinders that portion of the molecule, further inhibiting the lipid from the necessary interaction with the toxicity receptor.

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[2]Hristove and Needham, "Physical Properties of Polymer-Grafted Bilayers" in STEALTH LIPOSOMES, CRC Press, (1995) Chapter 5, pages 35-49; previously submitted.

[3]Noppl-Simson and Needham, *Biophys. J.*, 70(3):1391 (1996).

In summary, Applicants urge the Examiner to consider the entire teaching of Janoff *et al.* Applicants submit that if the document is considered as a whole, a modification of Janoff *et al.* to include a PEG chain at the head group of the lipid ligand would render the lipid ligand inoperable for its intended purpose. Accordingly, the cited references of Janoff *et* al. and Yoshioka *et* al. cannot be properly combined and do not render Applicants' invention obvious.

D. Analysis: Rejection over Popescu *et al.* in view of Yoshioka *et al.*

Applicants respectfully submit the Examiner has not appreciated the teaching of Popescu *et al.* as a whole. First, the Examiner asserts that Popescu *et al.* teach liposomes containing gentamycin. Second, the Examiner notes that Popescu *et al.* teach treatment of infection caused by *Listeria spp.* (Col. 5, line 11) and that because *Listeria ssp.* is treatable by Applicants' invention (page 42 of specification), the sites of infection must be the same.

With respect to the Examiner's first assertion, Applicants urge the Examiner to realize that Popescu *et al.* describe a liposomal composition for treating infections which reside in the RES, specifically in macrophages (Col. 4, lines 49-64). As such, the composition described by Popescu *et al.* is intended to be, after *in vivo* administration, taken up by phagocytic cells of the RES (Col. 5, lines 2-10).

In contrast, the liposomes of the present invention are intended to avoid uptake by the RES, in order to concentrate the liposomes at a site of tissue infection. A modification of the liposomes in Popescu *et al.* to include PEG-derivatized phospholipids of Yoshioka *et al.*, would prevent the liposomes from being taken up by the RES, thereby defeating the intended purpose of the composition in Popescu *et al.*

With respect to the Examiner's second point, Applicants do not understand its relevance. The claims at issue are not specific to *Listeria* ssp. Both the instant application and Popescu *et al.* disclose treatment of *Listeria* ssp. However, an infection caused by *Listeria* ssp. is presumably treatable by a variety of compositions and approaches, since the invasion process involves adherence to

intestinal cells and later uptake by the RES (see attached pages 182-189 from Salyers and Whitt[4]).

Importantly, the Examiner ignores very pertinent sections of Popescu *et al.* which speak to the intent and purpose of the composition in Popescu *et al.* Specifically at Col 4., lines 49-5 state:

> "In one scheme, SPLVs [small plurilamellar vesicles] are used to deliver therapeutic agents to sites of intracellular infections. Certain diseases involve an infection of cells of the reticuloendothelial system, *e.g., brucellosis*. These intracellular infections are difficult to cure for a number of reasons (1) because the infectious organisms reside within the cells of the reticuloendothelial system, they are sequestered from circulating therapeutic agents which cannot cross the cell membrane..." [brackets added].

At Col. 4, beginning at line 65:

> " According to one mode of the present invention, SPLVs containing an appropriate biologically active compound are administered (preferably intraperitoneally or intravenously) to the host organism or potential host organism... *Since phagocytic cells internalize SPLVs*, the administration of an SPLV-encapsulated substance that is biologically active against the infecting agent organism will result in directing the bioactive substance to the site of infection." (*emphasis added*)

Applicants respectfully urge the Examiner to reconsider the current rejection and to consider the teaching of Popescu *et al.* as a whole. Any modification of Popescu *et al.* in accord with the teaching of Yosioka *et al.* would alter the liposome composition of Popescu *et al.* in such a way that it would no longer fulfill its intended purpose.

Accordingly, Applicants respectfully request withdrawal of the rejections under 35 U.S.C. §103.

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[4]Salyers, A. and Whitt, D, BACTERIAL PATHOGENESIS: A MOLECULAR APPROACH, ASM Press, (1994).

VI. Conclusion

In view of the above remarks, Applicants submit that the claims now pending are in condition for allowance. A Notice of Allowance is, therefore, respectfully requested.

The Examiner is invited to contact Applicants' representative at the below-listed number if it is believed that prosecution of this application may be assisted thereby.

Respectfully submitted,

Date: 4/6/00

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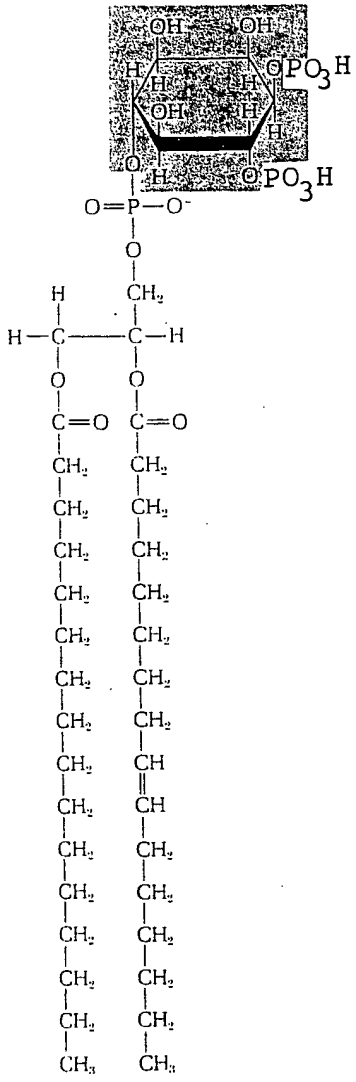
ATTACHMENT 1



Phosphatidylinositolbisphosphate

Bacterial Pathogenesis

A MOLECULAR APPROACH

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15

Listeria monocytogenes

Listeriosis

Listeria monocytogenes is a gram-positive, facultative, highly motile rod that causes an uncommon but potentially serious type of food-borne infection. As can be seen from Table 11-1, *L. monocytogenes* is notable among food-borne pathogens for the percent of cases that result in fatalities, exceeding even *Clostridium botulinum*. Most of the deaths caused by *L. monocytogenes* involve fetuses, newborns, or immunocompromised people. *L. monocytogenes* is normally found in soil, in water, on plants, and in sewage. It can also be isolated from many domestic animals and causes occasional disease in cattle and sheep. During the past decade, there have been several large outbreaks of human listeriosis, all of which were associated with commercially sold foods. Listeriosis is commonly associated with foods containing milk, but many types of foods, ranging from fresh vegetables and coleslaw to paté and shrimp, have been implicated in outbreaks. Outbreaks have also occurred in hospitals with large numbers of immunocompromised patients. Outbreaks involving multiple cases have received most of the public attention, but there are some indications that sporadic cases of listeriosis also occur and may be more common than has been previously appreciated.

In the average, healthy adult, *L. monocytogenes* infections are usually asymptomatic or at most produce mild influenzalike symptoms. Less commonly, diarrhea and abdominal discomfort can occur. In fact, *L. monocytogenes* is carried in the intestinal tracts of 5% to 10% of the population without any apparent symptoms. *L. monocytogenes* causes more serious infections

in adults with underlying conditions that compromise their immune responses (AIDS, cancer, diabetes, old age, and alcoholism). In such people, listeriosis can cause central nervous system infections (encephalitis, meningitis) and fatal bacteremia. *L. monocytogenes* is one of the few bacteria that can cross the placenta, which usually acts as a very effective filtration barrier to prevent blood-borne pathogens from gaining access to the fetus. In pregnant women who contract listeriosis, the bacteria can (but do not always) infect the fetus, resulting in stillbirths, preterm labor, or an infant born alive but with a systemic *L. monocytogenes* infection. To make matters worse, pregnancy seems to increase a woman's susceptibility to infection in the first place.

Virulence Factors

Animal and Cell Culture Model Systems

L. monocytogenes causes a systemic infection in mice, and this animal model system is used to assess virulence of mutants. Pregnant mice injected intraperitoneally with *L. monocytogenes* are used to study transplacental transmission of the bacteria. If pregnant gnotobiotic mice are used, *L. monocytogenes* can be introduced by the oral route to achieve the same end, thus providing a more natural model for listeriosis that results in fetal infections. The pregnant mouse models are still quite new, and virtually no molecular studies have been done with them; however, the existence of such models now makes it possible to test potential virulence factors identified in tissue culture model systems for their contribution to transplacental transfer of *L. monocytogenes*.

Murine listeriosis has long been used as a model system for studying the cell-mediated response to bacteria. The activated macrophage response was first discovered using this model system. Murine listeriosis has continued to produce new insights into how the body copes with intracellular bacterial pathogens and has led to the realization that some cell-mediated defenses previously thought to be specific for viral infections (e.g., natural killer cells and cytotoxic T cells) are also important for the control of intracellular bacterial pathogens such as *L. monocytogenes*.

Anatomy of a Listeriosis Outbreak: The Insidious Nature of the Disease*

Case 1. In 1981, an outbreak involving 41 people occurred in Canada. Thirty-four of the cases involved perinatal infections. There were 9 stillbirths and 23 infants born infected, of whom nearly one third died. Two pregnant women with symptomatic listeriosis delivered live healthy infants. Of the 77 nonpregnant adults who developed full symptomatic disease, nearly 30% died. The source of the outbreak was coleslaw produced by a local manufacturer. One of the farmers who supplied cabbage to the manufacturer had had two sheep die of listeriosis. Sheep manure was used to fertilize the cabbage field. This could have been the source of *L. monocytogenes* on the cabbage. Harvested cabbages were stored in a large cold storage shed until taken to the processing plant. *L. monocytogenes* is one of the few bacteria that grows at cold temperatures, so cold storage allowed the bacteria to grow to higher numbers.

Case 2. In 1985, in California, 142 people developed symptomatic listeriosis. Of these, 93 were perinatal and 49 were adult cases. Thirty fetuses or newborn infants died, and 18 adults died. Of the 49 adult cases, 48 occurred in people who were immunocompromised or elderly. The source of the bacteria was a certain brand of soft cheese. The plant in which the cheese was produced was ostensibly operated in compliance with safety regulations, and pasteurized milk was used to make the cheese. What appears to have happened is that on some occasions the pasteurizing equipment could not keep up with the flow of raw milk coming into the plant, and raw milk may have gotten into the final product.

*Adapted from A. Schuchat, B. Swaminathan, and C. V. Broome. 1991. Epidemiology of human listeriosis. Clin. Microbiol. Rev. 4:169–183.

L. monocytogenes readily invades intestinal cell lines (e.g., Caco-2 cells) and macrophages, and these cell lines are used for in vitro studies of invasion and actin rearrangement. Like *Shigella* spp., *L. monocytogenes* makes "plaques" (zones of killing) on fibroblast monolayers, and the same sort of plaque assay is used to quantitate cell-to-cell spread of *L. monocytogenes* as is used for *Shigella* spp. An exciting new development is an in vitro system in which *L. monocytogenes* polymerizes actin and moves for hours in a cell-free extract from *Xenopus* oocytes. This will allow a detailed biochemical analysis of the actin polymerization phenomenon already mentioned in connection with *Shigella* spp., which appears to contribute to cell-to-cell spread of *Shigella* spp., *L. monocytogenes*, and possibly other bacterial pathogens as well.

Genetic System

L. monocytogenes, being gram positive, is not closely related to the *E. coli* group and until recently could not be genetically manipulated. In general, genetic manipulation of gram-positive bacteria has lagged far behind genetic manipulation of gram-negative bacteria. This problem has been solved in part by using *Bacillus subtilis* instead of *E. coli* for cloning and expressing genes not well expressed in *E. coli*. Also, derivatives of the conjugative transposon Tn*916* (see Chapter 6), including Tn*917* and Tn*917-lac* (a derivative that allows random generation of *lacZ* fusions) are proving useful for mutagenesis and gene expression studies. Another important development is the ability to make gene replacements so that mutations can be easily introduced into chromosomal genes. Given the problems that had to be overcome to establish a genetic system, it is impressive how much people working in this area have accomplished. *L. monocytogenes* continues to compete very successfully with *Shigella* spp. as a model system for studying bacterial invasion. *L. monocytogenes* has a major advantage over *Shigella* spp. as a paradigm for invasive infections: *L. monocytogenes* infects mice.

Motility

L. monocytogenes is acquired by ingestion and must find and adhere to the intestinal mucosa. Thus, it was thought at first that motility due to flagella might be an important virulence factor. In fact, *L. monocytogenes* has peritrichous flagella, but the bacteria are motile only at temperatures considerably less than those found in the body (20°C to 25°C). At 37°C, production of flagella is much decreased, and it is likely that motility due to flagella is not important as a virulence factor. Another type of motility is clearly important for virulence: the ability of the bacteria to use host cell actin to move themselves within and between host cells. This type of motility has already been described in the case of *Shigella* spp. An elegant experiment was done to determine whether the actin tails of *L. monocytogenes* remained stationary or moved with the bacteria. A photoreactive compound was incorporated in growing actin tails, and then a section of a tail was pulsed with a thin beam of laser light (thus marking a discrete segment of the tail). From watching whether the band of light moved or remained stationary relative to the bacteria, scientists concluded that the actin tail remained stationary as the bacteria continued to move, proving that growth of the tail by actin polymerization at the end of the bacteria propels the bacteria through the cytoplasm.

Adherence and Invasion

L. monocytogenes attaches to and invades cultured mammalian cells. Steps in the invasion process and the virulence factors thought to be involved at each step are illustrated in Figure 15-1. Virtually nothing is known about the adherence step, although it is thought that α-D-galactose residues on the surface of the bacteria bind to α-D-galactose receptors on the intestinal cells. If so, this is the reverse of the situation seen in most cases of bacterial adherence where a protein on the bacteria binds a host cell carbohydrate moiety. As with *Shigella* spp., the bacteria are taken up by induced phagocytosis. An 80-kDa membrane protein called **internalin** may be involved in invasion. A tranposon insertion in ***inlA***, the gene encoding internalin, eliminates the ability to invade cultured cells. *inlA* is part of an operon that also contains a downstream gene, *inlB*, of unknown function. Thus, the insertion in *inlA* could have had a polar effect on *inlB*, (i.e., *inlB* could be essential for invasion). This appears not to be the explanation for the noninvasive phenotype, however, because a clone carrying only *inlA* complemented the mutation in *inlA* to full invasiveness. InlA shares amino acid sequence similarity with the M protein of *Streptococcus pyogenes*, a cell surface protein that prevents complement activation and is therefore antiphagocytic. Thus, at first glance, InlA (which stimulates phagocytosis) appears to have the opposite function from its homolog in *S. pyogenes*. This difference could be misleading, however, if the two proteins prove to interact in similar ways with host proteins that have shared domains but dissimilar functions. The fact that the receptor for InlA on macrophages appears to be a complement receptor and that M protein binds serum factor H and C3b could be pointing to such a connection. In *L. monocytogenes*, a single protein appears to be sufficient

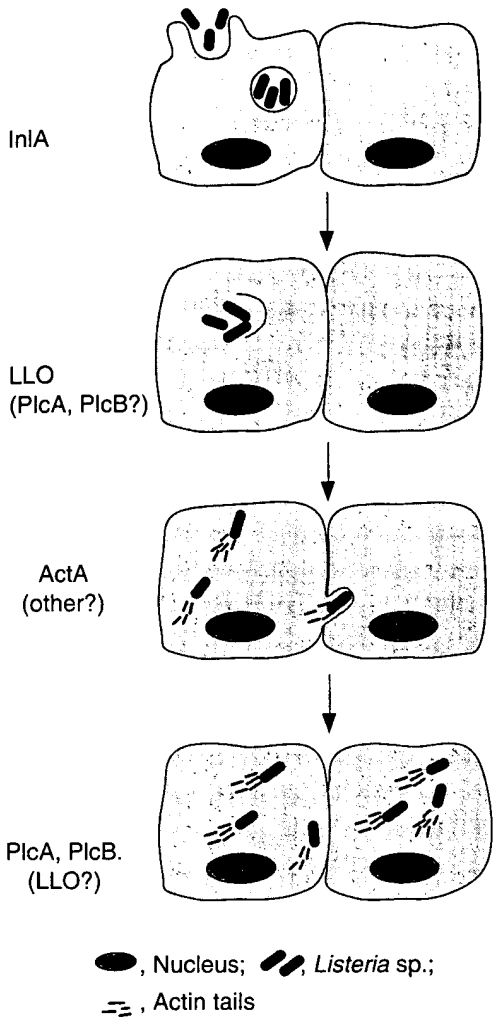

Figure 15-1 Steps in the invasion of cultured cells and intracellular spread by *L. monocytogenes*. The virulence factors thought to be involved at each step are indicated. "?" means that there is some uncertainty about the importance of the particular factor for that step. InlA is probably not necessary for ingestion of the bacteria by macrophagelike cell lines, that are naturally phagocytic, but is essential for invasion of cell lines which are not normally phagocytic.

for a process that required three proteins (IpaB–D) in *Shigella* spp. Thus, *L. monocytogenes* could be a better model for studying invasin function than *Shigella* spp. because its invasion process is simpler.

L. monocytogenes, like *Shigella* spp., will invade undifferentiated tissue culture cells but not differentiated cells. In humans and animals, *L. monocytogenes* is clearly an invasive organism that leaves the intestine and crosses the mucosa to reach underlying tissue. Either *L. monocytogenes* uses M cells as an entry point (as appears to be the case for *Shigella* spp.), or it can enter intestinal crypt cells, which are the only undifferentiated mucosal cells. Nothing is known about how *L. monocytogenes* enters the body of an intact animal.

Listeriolysin O (LLO; *hly*)

Forced phagocytosis brings *L. monocytogenes* into the host cell encased in a vesicle membrane. The bacteria escape from the vesicle and multiply in the cell cytoplasm. Like *Shigella* spp., *L. monocytogenes* produces an extracellular protein that causes the vesicle to rupture. Whereas nothing is known about how the *Shigella* protein that catalyzes this activity functions, a considerable amount is known about how *L. monocytogenes* exits the vesicle. The ability of *L. monocytogenes* to escape a phagocytic vesicle is due to production of a hemolysin, **listeriolysin O (LLO).** LLO is responsible for the zone of β-hemolysis seen when *L. monocytogenes* is grown on blood agar plates. LLO is a 60-kDa sulfhydryl-activated, pore-forming cytotoxin, which has considerable amino acid sequence similarity to hemolysin-cytotoxins produced by two other gram-positive pathogens, *S. pyogenes* (streptolysin O) and *Streptococcus pneumoniae* (pneumolysin). The gene encoding LLO has been named ***hly***, for "hemolysin." LLO is a major virulence factor of *L. monocytogenes*. LLO^- mutants have an LD_{50} for mice that is five logs higher than that of the wild type. LLO^- mutants also do not survive in macrophages. This suggests survival of *L. monocytogenes* in macrophages could be due to its ability to escape the phagosome before phagolysosomal fusion occurs. *L. monocytogenes* also produces catalase and superoxide dismutase, two enzymes that could help to protect it from the oxidative burst in the phagolysosome, but the contribution of these enzymes to virulence is not known. The importance of LLO for escape from the phagocytic vesicle was shown by an experiment in which *hly* was expressed in *B. subtilis*. The *B. subtilis* strain was incubated with a macrophagelike cell line (to bypass the need for an invasin) to determine whether it could escape the phagocytic vesicle. Although wild-type *B. subtilis* remained inside the phagocytic vesicle, *B. subtilis* expressing LLO escaped the vesicle and entered the cytoplasm.

Phospholipases (PI-PLC, *plcA*; PC-PLC, *plcB*)

L. monocytogenes produces at least two other hemolysins besides LLO: **phosphatidylinositol-specific phospholipase C (PI-PLC)** and **phosphatidylcholine-specific phospholipase C (PC-PLC).** PC-PLC has now been shown not to be specific for phosphatidylcholine

but will probably retain its original name for convenience. Unlike LLO, which lyses host cells by forming pores in the membrane, the phospholipases disrupt host cell membranes by hydrolyzing membrane lipids such as PI and PC. PI-PLC (***plcA***) was originally reported to be a virulence factor because a mutant with an insertional disruption in *plcA* was unable to cause listeriosis in normal mice. Also, this mutant formed small plaques on a fibroblast monolayer, indicating that PI-PLC might aid intracellular spread of *Listeria*. However, a more careful analysis of this mutation has revealed that the effects of the mutation were caused by a polar effect on a downstream gene, ***prfA***. PrfA is a regulatory protein that appears to activate a number of genes, including *hly* and *mpl/actA/plcB* (see later discussion). Loss of expression of these genes would be expected to have a negative impact on virulence. Thus, the importance of *plcA* itself is unclear. It may simply be providing *prfA*, the next gene in the operon, with a promoter.

Because phosphatidylcholine is also called lecithin, the second phospholipase (PC-PLC) is sometimes referred to as lecithinase. The gene encoding PC-PLC has been designated ***plcB***. PC-PLC has amino acid sequence similarity to lecithinases produced by two other gram-positive species, *C. perfringens* and *B. cereus*. The *C. perfringens* enzyme is thought to be an important virulence factor that contributes to tissue damage in wound infections caused by this pathogen, but the type of extensive tissue damage seen in *C. perfringens* wound infections is not seen in listeriosis. Because mutants lacking PC-PLC produce small plaques on fibroblast monolayers, PC-PLC could instead be contributing to cell-to-cell spread of *L. monocytogenes*.

A gene encoding a **Zn-dependent metalloprotease (*mpl*)** is near *plcB* on the chromosome of *L. monocytogenes*. Transposon insertions in this locus reduce virulence. The mutants not only fail to make the metalloprotease but also fail to make PC-PLC. This could be due to a polar effect. However, the mutants make a protein that is somewhat larger than mature PC-PLC (33 vs. 29 kDa) but cross-reacts with antiserum to PC-PLC. This result suggests that Mpl has some role in processing PC-PLC. Thus its contribution to virulence may be indirect.

Actin Tails

In the host cell cytoplasm, *L. monocytogenes* multiplies rapidly. It has been estimated that the bacteria inside a host cell divide once every 50 min, a high rate of growth for an intracellular pathogen. Apparently *L. monocytogenes*, like *Shigella* spp., finds the host cell cytoplasm a very congenial environment. *L. monocytogenes* moves through the cytoplasm to invade adjacent cells by polymerizing actin to form long tails, similar to *Shigella* spp. *L. monocytogenes* can move as rapidly as 1.5 μm/sec using this mode of locomotion. The tails are composed of short actin fibrils and extend from only one end of the bacterium. The shortness of the actin fibers could be the result of competition between bacteria and host cell factors for unpolymerized actin. The actin tail is actually a hollow mesh that forms on the surface of the bacterium and is left behind as the bacterium moves forward. With time, the actin filament mesh depolymerizes because of normal host cell actin turnover mechanisms. Thus, the tail gradually disappears as the bacteria move further along. The actin nucleating factor secreted by the bacteria is thought to be a protein but has not yet been isolated and characterized. A transposon insertion in a gene encoding a 90-kDa protein abolishes actin tail formation in macrophages. The gene has been designated ***actA***, but it is still not clear whether this protein is the actin nucleator. Evidence suggesting that ActA is, in fact, involved in actin tail formation is the recent finding that ActA, like IcsA of *Shigella* spp., is localized at one end of the bacterium, as expected from the fact that the actin enucleation occurs preferentially at one end of the rod-shaped bacteria. Also, ActA is not found in the tail, indicating that whatever ActA does, it is not shed along with the growing actin tail.

When the bacteria encounter the host cell plasma membrane that separates the host cell initially invaded from adjacent cells, they continue to move forward, producing protrusions that extend into the adjacent cell. The bacteria somehow escape the protrusions to enter the cytoplasm of the adjacent cell. Because mutants lacking PC-PLC have the small plaque phenotype, this phospholipase may play a critical role in the process by which the bacteria penetrate the membranes that initially wall them off from the adjacent cell.

Other Possible Virulence Factors

An operon called ***lmaBA*** encodes a 20-kDa protein, LmaA, which is localized on the bacterial surface. This protein induces delayed hypersensitivity when injected into mice that have been immunized against *L. monocytogenes*. That is, LmaA elicits a cell-mediated response to the bacteria. The role, if any, of LmaA in virulence is unknown. The same is true for LmaB, a 14-kDa protein of unknown function. *L. monocytogenes* also produces a 10-kDa protein that can remove iron from transferrin, a host iron-binding protein. This process requires NADH and Mg^{2+}, but little is known about the mechanism of iron removal or whether the protein is essential for virulence.

Systemic Spread of *L. monocytogenes*

In mice infected with *L. monocytogenes*, the bacteria first appear in macrophages and then invade hepatocytes. Most of their replication probably occurs in the liver. Because the bacteria are growing intracellularly in hepatocytes, a cell-mediated host response that kills infected cells is important for eliminating the bacteria. It has been shown that infection of macrophages with *L. monocytogenes* leads to presentation of bacterial antigens in a complex with MHC class I, thus stimulating the cytotoxic T cell response. Cytotoxic T cells (and natural killer cells, another type of phagocytic cell that recognizes infected host cells) may help to kill the infected hepatocytes. Bacteria released from these lysed host cells could then be killed by activated macrophages, which are able to kill *L. monocytogenes*. T cell–deficient mice are able to survive *L. monocytogenes* infection, so although the cytotoxic T cell response may help clear hepatocytes infected by bacteria, this response is not essential. Mice lacking the ability to produce interferon-gamma (IFN-γ) are more susceptible to *L. monocytogenes* than other mice, a fact that probably reflects the importance of activated macrophages as part of the host response, because IFN-γ stimulates macrophage activation. A failure of the host's cell-mediated response to control *L. monocytogenes* allows the bacteria to spread systemically. The placenta is largely composed of endothelial cells. The ability of *L. monocytogenes* to cross the placenta could be due to its ability to invade these cells and move from cell to cell, but this possibility remains speculative. Nothing is known about the ability of *L. monocytogenes* to invade endothelial cells.

Organization and Regulation of Virulence Genes

Listeria virulence genes are located on the chromosome, not on a plasmid, as was the case with *Shigella* spp. Many of the *L. monocytogenes* virulence genes are, however, clustered in the same region of the *L. monocytogenes* chromosome (Figure 15–2).

The genes *mpl*, *actA*, *plcB*, and three open reading frames of unknown function appear to be organized in an operon, although Northern blot analysis indicates that there may be more than one promoter in this region, in particular, a promoter downstream of *mpl*. Thus, insertions in *mpl* appear to be only partially polar on *actA* and other downstream genes. *plcA* is part of an operon that contains *prfA*. As mentioned earlier, an insertion in *plcA* was polar on *prfA*. *prfA* is thought to encode a positive regulatory protein that activates transcription of itself, *plcA*, *hly*, and the *mpl/actA/plcB* operon. There are three lines of evidence for this. First, mutations that disrupt *prfA* drastically reduced expression of *hly* and the other genes in this region. Second, providing *prfA* on a multicopy plasmid (i.e., overexpressing *prfA*) led to elevated expression of *hly* and the other genes in this region. Third, a cloned copy of *prfA* activated transcription of an *hly-lacZ* fusion in *B. subtilis*. Genes controlled by *prfA* are thermoregulated (i.e., expressed at higher levels at 37°C than at lower temperatures). Thus, PrfA may be sensing temperature.

Treatment and Prevention

Antibiotic treatment of pregnant women or immunocompromised people who have eaten food contaminated by *L. monocytogenes* can prevent the most serious consequences of the disease if the infection is diagnosed in time. Usually it is not diagnosed early enough. Often the first signs of an outbreak are cases of stillbirths or serious infections in susceptible adults. Although the most publicized cases of listeriosis are those that involved large outbreaks, sporadic cases of listeriosis also occur. Because cases of listeriosis are frequently associated with commercially produced foods, avoiding contamination of foods in the first place would be the ideal solution. This is not yet feasible.

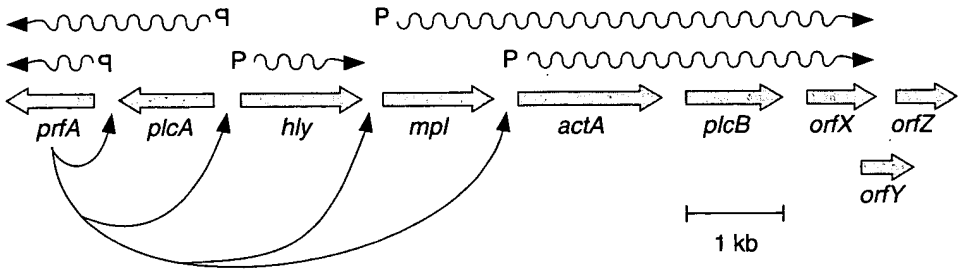

Figure 15-2 Organization and regulatory control of some *L. monocytogenes* virulence genes that are clustered on the chromosome. *prfA*, regulatory protein (activator); genes activated by PrfA are indicated by arrows: *plcA*, PI-PLC; *plcB*, PC-PLC (lecithinase); *hyl*, LLO; *actA*, protein involved in actin polymerization (?); *orfX*, *Y*, *Z*, orfs of unknown function; probable locations of promoters are indicated by "P"; wavy lines indicate mRNA transcripts.

Tests that are rapid and simple enough to be done on a large scale by food processors are not yet available, although attempts to develop such tests are currently underway. Another complication is that *L. monocytogenes* is able to grow at low temperatures. Thus, refrigeration is not as effective in preventing growth of *L. monocytogenes* in food as it is for most other bacteria that cause food-borne disease. Add to this the fact that at least 5% to 10% of the adults in the population are asymptomatic carriers of *L. monocytogenes*, and it is easy to see why *L. monocytogenes* is a food processor's nightmare.

For the present, prevention is in the hands of the food consumer. Pregnant women and immunocompromised people are advised to take the following precautions. Avoid all soft cheeses and do not consume unpasteurized milk. Thoroughly cook all meats, especially processed meat products such as hot dogs. Because *L. monocytogenes* grows in the refrigerator, all leftover meats should be reheated until steaming hot before they are eaten. This same precaution applies to deli meats and cold cuts purchased from the store. Raw vegetables should be washed thoroughly before being eaten.

The possibility of a vaccine is not being seriously considered for the same reason a vaccine for botulism is not practical: The number of cases is too small.

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SUMMARY

1. Listeriosis is caused by *L. monocytogenes*, a highly motile, gram-positive, facultative rod found in soil, water, plants, sewage, and domestic animals. It is carried in the gastrointestinal tract of 5% to 10% of the human population. Listeriosis is usually either asymptomatic or at worst a mild influenzalike illness in healthy adults but can be a serious, fatal disease in immunocompromised people. *L. monocytogenes* is one of the few bacteria that can cross the placenta. In pregnant women, it can cause stillbirths, preterm labor, or systemic infections in the infant after birth. During the past decade there have been several large outbreaks of listeriosis related to commercially prepared foods, especially those containing milk.

2. *L. monocytogenes* causes a systemic infection in mice. The genetic system available for *L. monocytogenes* is not as sophisticated as that for gram-negative species, but it has been developing rapidly and is now sufficiently advanced to allow in-depth molecular analysis to be done.

3. *L. monocytogenes* has flagella and is highly motile at low temperatures. However, production of flagella is probably not an important virulence factor in the human body. A second type of motility due to actin rearrangement clearly plays an important role in the ability of these bacteria to spread from cell to cell. The mechanism by which *L. monocytogenes* attaches to the host cell is not well understood but probably involves attachment of an α-D-galactose residue on the bacterial surface to a protein receptor on the host cell. This is the reverse of the usual attachment process in which a bacterial protein recognizes a host cell carbo-

hydrate. Once bound to a host cell, *L. monocytogenes* (like *Shigella* spp.) induces phagocytosis. Invasion is mediated by internalin, a membrane protein with amino acid similarity to M protein of *S. pyogenes*.

4. A major virulence factor is the surface protein listeriolysin O (LLO), which is a sulfhydryl-activated, pore-forming cytotoxin (hemolysin). LLO allows the bacteria to escape from the phagocytic vesicle. LLO has amino acid similarity to streptolysin O (*S. pyogenes*) and pneumolysin (*S. pneumoniae*). LLO is encoded by the *hly* gene.

5. *L. monocytogenes* produces at least two phospholipases that disrupt host cell membranes by hydrolyzing membrane lipids. Phosphatidylinositol-specific phospholipase C (PI-PLC, *plcA*) was initially thought to be important for virulence, but an insertional mutation that led to this conclusion has now proved to exert its effect by polarity on a downstream gene. Phosphatidylcholine-specific phospholipase C (PC-PLC, *plcB*), a lecithinase, probably contributes to cell-to-cell spread. PC-PLC may be processed by Mpl, a metalloprotein.

6. *L. monocytogenes* moves through the host cell cytoplasm to invade adjacent host cells by polymerizing actin to form long actin tails. Actin tail formation is catalyzed by ActA, a protein that is localized on the bacterial surface near one end of the bacteria, where the growing actin tail is forming.

7. In contrast to *Shigella* spp., the *L. monocytogenes* virulence genes are located on the chromosome, but many are clustered in the same region of the chromosome. Some of the genes (*mpl*, *actA*, *plcB*, and others of unknown function) are arranged in an operon. Another operon contains *plcA* and *prfA*. PrfA is a positive regulator that activates transcription of itself, *plcA*, *hly*, and *mpl/actA/plcB*. PrfA may sense temperature.

8. Early antibiotic treatment can prevent the most serious consequences of human listeriosis, but early detection of listeriosis is rare. The food industry is working to develop tests to monitor certain types of food commonly associated with outbreaks for contamination by *L. monocytogenes*. Refrigeration is not helpful because *L. monocytogenes* grows at low temperatures. Avoiding soft cheese, careful washing of vegetables, and thorough cooking of food, including reheating of leftovers, can help to prevent cases of listeriosis in pregnant women and immunocompromised people.

QUESTIONS

1. Why was it so important to find a transposon that could be used to mutagenize *L. monocytogenes*? Why not just use chemical mutagenesis, as researchers have done for years to obtain mutants of *E. coli* and *Salmonella typhimurium*?

2. How could an actin structure such as that produced by *L. monocytogenes* cause movement of the bacteria?

3. A transposon insertion in *actA* stops tail formation by *L. monocytogenes*. Does this prove that ActA is an actin nucleator? What are the other possibilities?

4. Speculate about why listeriosis is so different from dysentery in its symptoms and progression despite the fact that *L. monocytogenes* and *Shigella* spp. seem to be so similar in their invasion of cultured cells and their intracellular behavior.

5. How do *L. monocytogenes* and *Shigella* spp. differ with respect to gene organization?

6. Infection with *L. monocytogenes* produces an antibody response, but this response is not protective. Explain why this is so.